

Regulation of the glutamate-dependent acid-resistance system of diarrheagenic *Escherichia coli* strains

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Abstract

The ability to withstand an acid challenge of pH 2.5 or less by *Escherichia coli* strains is a trait generally believed to be restricted to their stationary phase of growth. Of the three distinct acid-resistance systems that have been identified in *E. coli*, the glutamate-dependent acid resistance (GAD) system provides the highest level of acid resistance. Earlier reports indicated that in the exponential growth phase of *E. coli* K-12 strains the GAD system is not active. The present study reports that when grown on minimal medium several diarrheagenic and K-12 strains of *E. coli* have a complete set of induced genes necessary for GAD in the exponential growth phase to overcome the acid challenge of pH 2.5 for several hours. A previously identified factor(s) specific to the GAD system in the stationary phase and predicted to undergo dilution during the exponential phase appears to be glutamate-decarboxylase isozyme(s) inactivated differentially in the rich vs. minimal growth media.

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1. Introduction

Members of the family *Enterobacteriaceae* encounter low-pH stress during passage through the stomach en route to the intestine. Acid resistance (AR) is an important property of *Escherichia coli*, enabling the organism to colonize and establish a commensal relationship with mammalian hosts [1,2]. The low infectious dose associated with diarrheagenic *E. coli* serotypes is attributed to its acid-resistant nature [2–4]. Three distinct AR systems have been identified. *E. coli* cells grown aerobically to the stationary growth phase in Luria–Bertani broth (LB) develop AR which allows cells to survive exposure to pH 2.5 [3,5,6]. One factor critical to this system is the stationary-phase-associated sigma factor σ^S , the product of the *rpoS* gene [7,8]. Once the stationary phase AR system is induced, the manner in which it protects cells during acid challenge is

still unknown [9,10]. The *rpoS*-mediated AR system is not operative in fermentatively metabolizing cells (grown in complex medium containing glucose) [11,12]. Maintenance of neutral pH_i under fermentative growth conditions is achieved by the induction of two amino acid decarboxylation-antiporter systems [8,11,13]. One system requires glutamic acid during acid challenge, and the other requires arginine [14,15]. The net effect of the decarboxylation reactions is believed to be increasing the alkalinity of the cytoplasmic compartment [3,10,14].

The glutamate-dependent AR (GAD) system is believed to provide the highest level of AR [6,13]. The GAD system is comprised of at least three genes. Two of these genes, *gadA* and *gadB*, encode highly homologous glutamate decarboxylase isoforms. The third gene, *gadC*, encodes a glutamate:GABA antiporter. The confusion in the literature concerning when and how the *gad* genes are regulated stems partially from their control by multiple global regulators (i.e. cAMP-CRP, H-NS and RpoS), and data from various laboratories that differ in the use of complex versus minimal media, exponential growth phase versus stationary phase cells, and acid challenge at various pHs and durations [7,8,16–18]. To further complicate the understanding of the GAD system was the observation that,

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although glutamate decarboxylase isoforms are expressed highly in exponential growth phase cells of *E. coli* K-12 (at pH 5.5), the system was not functional and as a result cells were sensitive to acid challenge at pH 2.5 [6]. This observation prompted speculation that additional factors present in stationary phase cultures may be required for activation of the GAD system in vivo [3,6,12].

In this study, the GAD system from several diarrheagenic and K-12 *E. coli* strains was examined. It was observed that the cells have a fully functional GAD system in the exponential growth phase, which enabled cells to overcome the acid challenge of pH 2.5 for several hours. The regulation of the GAD system of *E. coli* strains was subjected to pH and the nature of growth media.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The strains of *E. coli* used are listed in Table 1. Frozen stocks maintained at -75°C were streaked on LB agar and after overnight growth at 37°C , a single colony was inoculated into minimal E medium [19] containing 0.4% glucose at pH 7.0 unless stated otherwise (EG minimal medium) [13], or in a complex growth medium of LB buffered with 100 mM MES (morpholineethanesulfonic acid, pH 5.5) or 100 mM MOPS (morpholinepropanesulfonic acid, pH 8.0). Most diarrheagenic *E. coli* strains are auxotrophs and required amino acids or vitamins for growth in minimal media [2,20]. The precise requirement for vitamins and amino acids for individual strains was not determined; instead, EG minimal medium was supplemented with 50 μg of yeast extract per ml. Strain EK274 is auxotrophic for thiamine, nicotinamide and riboflavin [20]. The strain had an identical GAD phenotype when grown in EG+vitamins and EG+yeast extract. Strain MG1655 is not an auxotroph and addition of yeast extract had no influence on its GAD phenotype. To obtain stationary phase cultures, cells were grown in the media on an orbital shaker (210 rpm, 37°C) for 20–22 h to an optical density of 600 nm (OD_{600}) at 2.8–3.0. To obtain exponential growth phase cells, overnight cultures were diluted 200-

fold in prewarmed (37°C) EG medium (of pH 5.5–7.8, as indicated) or in LB-MES medium (50- to 2000-fold) and grown for 6–7 h to an OD_{600} of 0.35–0.4.

2.2. Chloramphenicol treatment

The chloramphenicol was used at a concentration of 50 $\mu\text{g ml}^{-1}$ where bacteriostasis occurred and no lethal effect was observed in the *E. coli* strains mentioned in this study. The antibiotic was added 15 min before acid challenge as well as during acid challenge.

2.3. Acid-challenge assay

Cells were diluted directly from the growth media (1:20, 1:100 or 1:1000) to EG medium (pH 2.0 or pH 2.5) supplemented with glutamate (1.5 or 5 mM), which was prewarmed to 37°C . Viable counts were determined at 0, 1, 2 and 4 h after acid challenge by diluting cells in phosphate-buffered saline (50 mM, pH 7.2) and plating immediately on LB agar media.

2.4. Western blot analysis

Strains were grown at 37°C as indicated in EG or LB media. At an OD_{600} of 0.35 (exponential growth phase) or 3.5 (stationary phase), cells were collected by centrifugation ($12\,500\times g$, 4 min), resuspended at 1 OD_{600} unit ml^{-1} in $1\times$ loading buffer [50 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 2.5% β -mercaptoethanol, 0.01% Na-azide, 0.1% bromophenol blue]. Each protein extract was fractionated on SDS-polyacrylamide gradient gels (4–20%). After electrophoretic transfer of proteins onto nitrocellulose membranes, the glutamate decarboxylase isozymes were revealed by using rabbit primary antibody raised against synthetic polypeptide 439EDYKASLKYLS DHPKLQ455, corresponding to the sequence at the C-terminal end of GadA/B (Research Genetics, Huntsville, AL, USA), which was able to detect both isozymes. The anti-rabbit antibody raised in goat coupled to peroxidase was used as secondary antibody. SuperSignal chemiluminescent kit (Pierce Biotechnology, Rockford, IL, USA) was used to quantitate the primary

Table 1
E. coli strains used in this study

Strain	Serotype, genotype ^a or relevant information	Reference
<i>E. coli</i> MG1655K-12 wild-type		[5]
EK274	O157:H7 wild-type (ATCC 43895), Na ^r Rf ^r	[13]
EK275	EK274 <i>rpoS</i> :: <i>pRR10</i> Ap ^r	[6]
EF522	K12 <i>gadA</i> :: <i>pPR10</i> (Ap) <i>gadB</i> ::Km	[5]
EF362	K-12 <i>rpoS</i> :: <i>Tn10</i>	[6]
E2348/69	O127:H6, model EPEC strain (isolated from a child – 1969, Taunton, UK; model EPEC strain)	National Food Safety and Toxicology Center, Michigan State University, East Lansing, MI, USA)
G5506	O104:H21 (bloody diarrhea outbreak – 1994, MT, USA)	
Dec16A	O113:H21(diarrhea isolate – 1980, Bangkok, Thailand)	

^aAbbreviations for antibiotics: Ap, ampicillin; Km, kanamycin; Na, nalidixic acid; Rf, rifampicin.

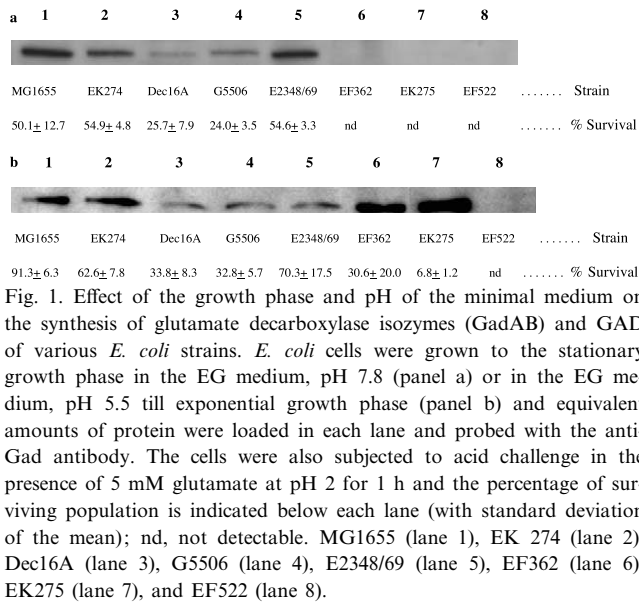


Fig. 1. Effect of the growth phase and pH of the minimal medium on the synthesis of glutamate decarboxylase isozymes (GadAB) and GAD of various *E. coli* strains. *E. coli* cells were grown to the stationary growth phase in the EG medium, pH 7.8 (panel a) or in the EG medium, pH 5.5 till exponential growth phase (panel b) and equivalent amounts of protein were loaded in each lane and probed with the anti-Gad antibody. The cells were also subjected to acid challenge in the presence of 5 mM glutamate at pH 2 for 1 h and the percentage of surviving population is indicated below each lane (with standard deviation of the mean); nd, not detectable. MG1655 (lane 1), EK 274 (lane 2), Dec16A (lane 3), G5506 (lane 4), E2348/69 (lane 5), EF362 (lane 6), EK275 (lane 7), and EF522 (lane 8).

antibody and signal was captured on preflashed Kodak X-ray film. The relative amounts of proteins were deduced from densitometric analysis of scanned films.

3. Results

3.1. GAD of exponential growth phase cells of *E. coli* O157:H7

Diarrheagenic and K-12 *E. coli* strains grown to stationary phase in complex and minimal media were examined for the GAD. Irrespective of the pH and the nature of growth media, in stationary growth phase the O157:H7 strain EK 274 was able to withstand acid challenge of pH 2.0 in the presence of 5 mM glutamate (Table 2, rows 1–

4). Also, as reported earlier [7,8,12] exponential growth phase cells grown in the LB medium had no GAD and the cells were sensitive to the acid challenge (Table 2, row 6). However, *E. coli* O157:H7 cells grown to the exponential growth phase in EG medium at pH 5.5 (but not at pH 7.8, see below) were resistant to the acid challenge of pH 2.0 for 1 h in the presence of glutamate (Table 2, row 7).

To enable direct comparison with previous observations [6,11,13], the acid-resistant phenotype of the exponential growth phase cells was examined at different dilutions and at varying lengths of time at pH 2.5 (Table 2, rows 8–11). Initial dilution of growth media as well as the duration of acid challenge for 2 h at pH 2.5 or 1 h at pH 2.0 had no significant effect on the measurement of the GAD phenotype (Table 2, rows 8 and 9 vs. row 7). It was further investigated whether active protein synthesis during the acid challenge was required by the exponential growth phase cultures to express the functional GAD system. Pre-treatment of cells with chloramphenicol as well as during acid challenge had no effect on the ability of cells to overcome acid challenge (Table 2, rows 12 and 13), indicating actively growing cultures have the full set of induced genes required to express GAD.

3.2. GAD of exponential growth phase cells of other pathogenic *E. coli* strains

Contrary to the data reported earlier for *E. coli* K-12 strains [6,12], the observation of the GAD in the exponential growth phase cells of *E. coli* O157:H7 strain EK274 (Table 2) prompted examination of other enteropathogenic *E. coli* isolates, namely, Shiga-toxin-producing (STEC) strains, Dec16A and G5506 (serogroup O113:H21 and O104:H21, respectively), and a model enteropathogenic *E. coli* (EPEC) strain E2348/69 (serogroup O127:H6). Further, synthesis of Gad isozymes was monitored by

Table 2
Measurement of glutamate-dependent AR of *E. coli* O157:H7 strain EK274

Row	Culture conditions		Acid-challenge parameters			% Survival ^a	
	Growth media	Growth phase	Final dilution from the growth media	pH	Duration (h)		Glutamate (mM)
1	LB-MOPS, pH 8.0	Stationary	1:20	2.0	1	5.0	86.7 ± 31.5
2	LB-MES, pH 5.5	Stationary	1:20	2.0	1	5.0	79.4 ± 11.2
3	EG, pH 7.8	Stationary	1:20	2.0	1	5.0	61.5 ± 9.2
4	EG, pH 5.5	Stationary	1:20	2.0	1	5.0	83.2 ± 7.3
5	EG, pH 7.8	Stationary	1:20	2.0	1	0.0	< 0.01
6	LB MES, pH 5.5	Exponential	1:20	2.0	1	5.0	< 0.01
7	EG, pH 5.5	Exponential	1:20	2.0	1	5.0	47.2 ± 2.9
8	EG, pH 5.5	Exponential	1:100	2.5	2	1.5	29.7 ± 4.9
9	EG, pH 5.5	Exponential	1:1000	2.5	2	1.5	50.7 ± 7.8
10	EG, pH 5.5	Exponential	1:100	2.5	4	1.5	49.1 ± 1.1
11	EG, pH 5.5	Exponential	1:1000	2.5	4	1.5	42.3 ± 5.9
12	EG, pH 5.5+Cm ^b	Exponential	1:20	2.0	1	5.0	57.6 ± 8.8
13	EG, pH 7.8+Cm ^b	Stationary	1:20	2.0	1	5.0	74.4 ± 9.6

^aAverage of at least three experiments, ± represents standard deviation of the mean.

^bCells were treated with chloramphenicol (50 µg ml⁻¹) 15 min prior to as well as during acid challenge.

Western blot analysis (Fig. 1). *E. coli* strain K-12 MG 1665 as well as other diarrheagenic strains in their stationary (pH 7.0) as well as exponential (pH 5.5) growth phase had Gad isozymes that could be detected by the Western blot analysis. Concomitantly, the cells were able to survive an acid challenge of pH 2.0 for 1 h in the presence of glutamate (Fig. 1).

3.3. Synthesis of GadA/B was independent of *rpoS* regulon

Synthesis and functioning of GAD in the stationary growth phase is regulated by the stationary-phase-associated sigma factor σ^S , the product of the *rpoS* gene. Therefore it was of interest to determine if GAD that is observed in the exponential growth phase cells is also regulated by RpoS. The *rpoS* mutant strains EK275 and EF362 in their stationary growth phase grown in EG medium (pH 7.8) were sensitive to the acid challenge and did not exhibit the GAD (Fig. 1a). However, the exponential growth phase cells of the *rpoS* mutant strain EF362 grown in EG medium (pH 5.5) were able to withstand the acid challenge of pH 2 in the presence of glutamate (Fig. 1b). The *rpoS* mutant strain EK275 showed a weaker GAD in the exponential growth phase and only 6% of the cells survived the acid challenge even though the strain expressed Gad isozymes at high level.

3.4. In the EG growth medium, stability of Gad was pH dependent

It was further determined whether the exponential growth phase induction of GAD was sensitive to the pH of EG medium. The EK274 cells from the stationary growth phase (EG medium, pH 7.0) were diluted into fresh EG medium with pHs ranging from 5.5 to 7.8. After the cells reached the exponential growth phase ($OD_{600} \sim 0.35$ – 0.4), the cultures were acid challenged in the presence of 5 mM glutamate at pH 2.0 for 1 h at 37°C (Fig. 2a). The induction of the GAD system was observed when the media pH was in the range of 5.5–7.1. At pH 7.3 and higher there was a sharp decline in the GAD. The synthesis of Gad isozymes was followed in the exponential growth phase cultures grown at pH 5.5–

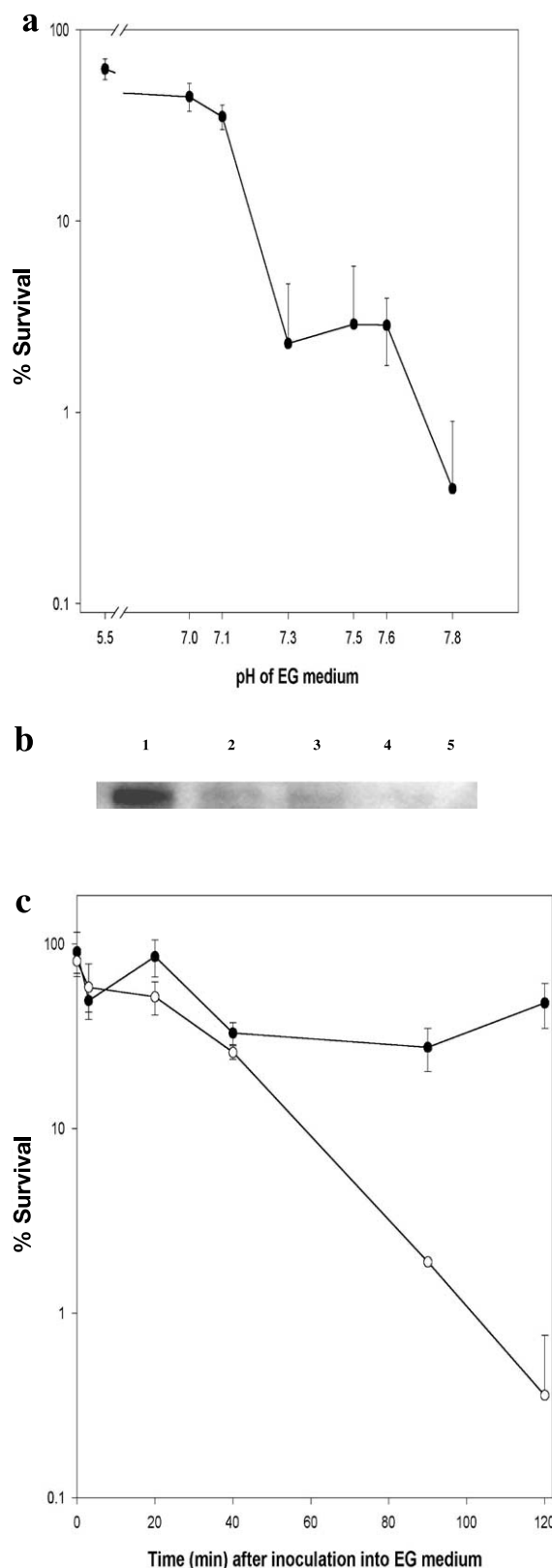


Fig. 2. Effect of pH of the minimal growth medium on GAD and on synthesis of GadAB. a: *E. coli* strain EK274 was subjected to acid challenge (pH 2 in the presence of 5 mM glutamate for 1 h) after it was grown to the exponential growth phase in EG medium with pH 5.5–7.8. b: Western blot analysis of cells from Fig. 2a, equivalent amounts of protein were loaded in each lane and probed with the anti-Gad antibody. Cells from minimal medium at pH 7.0 (lane 1), 7.3 (lane 2), 7.5 (lane 3), 7.6 (lane 4), and 7.8 (lane 5). c: GAD phenotype (acid challenge at pH 2 in the presence of 5 mM glutamate for 1 h) of cells during the first 120 min after stationary phase cells were transferred to fresh minimal medium at pH 7.8 (○) and pH 5.5 (●). Error bars represent the standard deviation (not shown if smaller than the symbol).

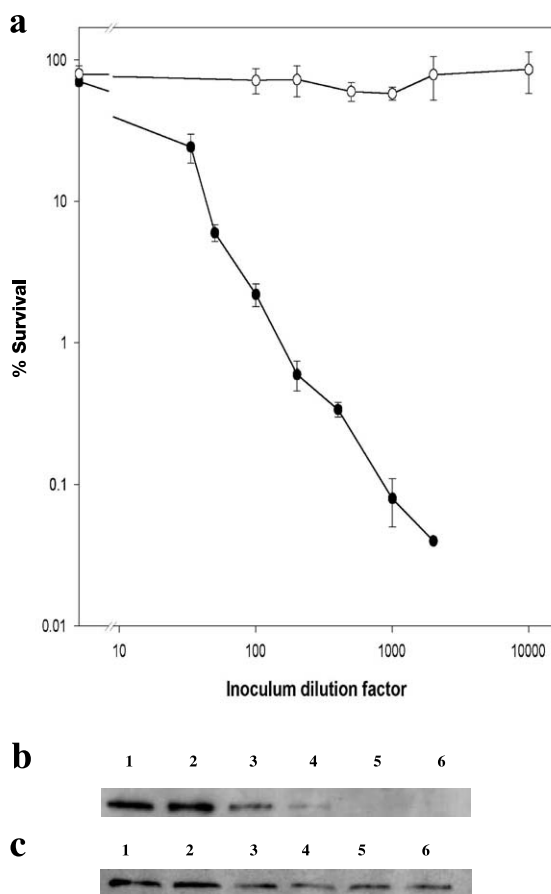


Fig. 3. The proportion of the starting inoculum in rich vs. minimal growth medium at pH 5.5 and its effect on GAD phenotype. a: varying quantities of stationary phase cells were inoculated into LB-MES (●) and minimal medium (○) and were grown to the exponential growth phase before subjecting them to acid challenge (pH 2 in the presence of 5 mM glutamate for 1 h). Error bars represent the standard deviation (not shown if smaller than the symbol). b: Western blot analysis with the Gad antibody of exponential growth phase cells from LB-MES medium with varying inoculum per 10 ml: 200 µl (lane 2), 100 µl (lane 3), 50 µl (lane 4), 25 µl (lane 5) and 10 µl (lane 6), and the stationary phase cells used as inoculum (lane 1); c: Western blot analysis with the Gad antibody of exponential growth phase cells from minimal medium with varying inoculum per 10 ml: 100 µl (lane 2), 50 µl (lane 3), 25 µl (lane 4), 15 µl (lane 5) and 10 µl (lane 6), and the stationary phase cells used as inoculum (lane 1).

7.8 (Fig. 2b). The exponential growth phase cells from EG medium of pH 7.3 and above had significantly less Gad isozymes as detected by Western blot analysis. Since the induction of GAD in the stationary growth phase cells at pH 7.8 was high (Fig. 1a), the GAD phenotype was compared during resumption of growth from the stationary growth phase to the exponential growth at pH 7.8 and 5.5 (Fig. 2c). Immediately after the transfer from stationary growth phase into the fresh EG medium, until first 40 min there was no change in the GAD response at pH 5.5 or at pH 7.8. However, at pH 7.8 cells lost their GAD by 90 min after inoculation into the fresh medium, while GAD system was still functional at pH 5.5 during lag

phase (Fig. 2c) and during stationary and exponential growth phase (Fig. 1a,b, respectively). There was no increase in the viable cell count during the first 90 min after inoculation irrespective of the pH of the EG medium (data not shown).

3.5. In the rich medium Gad was diluted regardless of pH

The induction of GAD by mildly acidic growth conditions was further examined in rich versus minimal growth media. It was observed that in rich media such as LB broth, the GAD of exponential growth phase cells was directly proportional to the inoculum dose (Fig. 3a), and Gad isozymes were not induced in spite of the low pH of the rich medium (Fig. 3b). On the other hand, inoculum dose had no influence on GAD of cells grown in EG medium at pH 5.5. The GAD and glutamate decarboxylase isozyme levels remained high in EG medium at pH 5.5 even though the starting inoculum ratio varied from 1:100 to 1:10 000 (Fig. 3a,c). The data indicate that the stationary phase cells (from the inoculum) continued to express genes required for GAD as they resumed growth in fresh minimal medium at pH 5.5.

4. Discussion

E. coli possesses exponential growth phase and stationary phase acid survival mechanisms [21–23]. While comparing the three AR systems of *E. coli* K-12 strain MG1665, Castanie-Cornet et al. [6] reported that induction of the glutamate decarboxylase isozymes is insufficient for GAD, based on the observation that exponential growth phase cells grown at pH 5.5 produced large amounts of GadA and GadB but failed to survive pH 2.5. This observation was interpreted as a possible need to process Gad proteins in stationary growth phase in order to be functionally active [3,12]. The data presented here indicate that the four diarrheagenic as well as K-12 strains of *E. coli* possess the complete set of induced genes for GAD to survive the acid challenge of pH 2.5 and below for several hours, provided glutamate is available during acid challenge.

In order to have a direct comparison with previous studies, the GAD phenotype was measured while taking into consideration various parameters such as cell density, glutamate availability, duration and pH during acid challenge (Table 2). The level of glutamate decarboxylase isozymes also was measured by Western blot analyses in four different pathogenic strains (Fig. 1). The observation that the cells taken from the exponential growth phase do not require de novo protein synthesis during acid challenge to exhibit a functional GAD system also suggests that the cells do not need additional factors from the stationary growth phase [3,6,12].

The expression of the GAD system was found to be

complex and varied according to whether cells were cultured in minimal or complex growth media [8,11,13]. The GAD system was functional in stationary phase cells regardless of pH and growth media. The acid induction of the GAD system in exponential growth phase cells was observed when cells were grown in minimal medium and the pH was below 7.1 (Figs. 1b and 2a). The repression of *gad* genes by CRP and H-NS has been documented [8,12]. The current evidence indicates that in complex growth medium such as LB broth, CRP-cAMP levels are high and RpoS is required for the induction of the GAD system [12]. The scenario typically is observed for the cells entering into the stationary growth phase. During growth in the minimal medium where cAMP levels are low, acid induction of *gad* genes in the exponential growth phase can be driven by housekeeping sigma factor (σ^{70}), the observation supported by the fact that the *rpoS* mutant strains EK275 and EF522 possess abundant Gad protein. However, the high Gad concentration was apparently not sufficient by itself to have a functional GAD system especially in the *rpoS* mutant strain EK275. The data probably indicate that RpoS regulon may be regulating the synthesis of additional genes (other than *gadA*, *gadBC*) or there may be additional regulatory elements influencing optimal functioning of the GAD system in the enteropathogenic strains of *E. coli* [24].

The stationary phase ‘factor’ reported by Small et al. [7] that persisted over several generations in the exponential growth phase appears to be glutamate decarboxylase (Fig. 3b). *E. coli* cultures grown at pH 5.5 in LB-MES broth to the exponential growth phase showed substantial AR when the initial dilution of the stationary phase culture was 50- to 100-fold (Fig. 3a and [7]). At greater dilutions, the GAD (and glutamate decarboxylase levels) of the exponential growth phase cells grown to the same OD₆₀₀ steadily declined (Fig. 3a,b). When the growth resumed from the stationary phase, glutamate decarboxylase isozymes were diluted by growth differentially in rich versus minimal medium. In rich medium, dilution was independent of pH (Fig. 3b), while in minimal medium the process was pH dependent (Figs. 2b and 3c).

In summary, several diarrheagenic and K-12 strains of *E. coli* possessed a complete set of induced genes necessary for GAD in the exponential growth phase to overcome the acid challenge of pH 2.5 for several hours. During the transition from the stationary growth phase to the exponential growth, glutamate decarboxylase isozymes were degraded in LB broth independent of pH, while in the minimal growth medium the degradation was pH dependent.

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